JOURNAL OF CLINICAL MICROBIOLOGY, Aug. 2009, p. 2577–2585 Vol. 47, No. 8
0095-1137/09/$08.00+0 doi:10.1128/JCM.00030-09

Antibody Responses to Recombinant Polyomavirus BK Large T and VP1 Proteins in Young Kidney Transplant Patients▽

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Received 7 January 2009/Returned for modification 1 May 2009/Accepted 20 May 2009

BK virus (BKV)-specific immunity is critical for polyomavirus-associated nephropathy, but antibody responses are incompletely defined. We compared the hemagglutination inhibition assay (HIA) with immunoglobulin G enzyme immunoassays (EIA) to BKV proteins expressed in baculovirus-infected insect cells. N-terminal, internal, and C-terminal domains of the BKV large T antigen (BKLT) were fused to glutathione *S*-transferase (GST), yielding GST-BKLTD1, GST-BKLTD2, and GST-BKLTD3, respectively. The BKV capsid VP1 was expressed as a GST fusion (BKVP1) or as a native VP1 assembled into viruslike particles (BKVLP). We tested 422 sera from 28 healthy donors (HD), 99 dialysis patients (DP; median age, 15 years; range, 3 to 32 years), and 46 age-matched kidney transplant patients (KTP; median age, 15 years; range, 2 to 33 years). In HD, HIA and BKVLP EIA both yielded a 91.7% seroreactivity, whereas all other EIA responses were lower (BKVP1, 83.3%; BKLTD1, 25%; BKLTD2, 29%; BKLTD3, 40%). HIA titers significantly correlated with EIA levels for BKVLP, BKVP1, and BKLTD1 but not for BKLTD2 or BKLTD3, which were barely above the cutoff. In DP, the seroreactivities of HIA, BKVLP, and BKLTD1 were lower than that in HD (63.6%, 86.9%, and 10.1%, respectively) and they had lower titers ($P < 0.001$). In KTP, seropositivities for BKVLP, BKVP1, and BKLTD1 were 78%, 50%, and 17%, respectively, but anti-BKVLP levels increased significantly in KTP with viruria and viremia, whereas anti-BKLTD1 levels increased after clearing sustained BKV viremia. In conclusion, anti-BKVLP is equivalent to HIA in HD but is more sensitive to determine the BKV serostatus in DP and KTP. In KTP, anti-BKVLP responds to recent BKV viruria and viremia, whereas anti-BKLTD1 may indicate emerging BKV-specific immune control.

Polyomaviruses (PyV) have been identified in many vertebrates; the main species found in humans are BK virus (BKV) and JC virus (C. Büchen-Osmond, *Polyomaviridae*, International Committee on Taxonomy of Viruses Database [www.ncbi.nlm.nih.gov/ICTVdb/]). PyV are small nonenveloped double-stranded DNA viruses with icosahedral particles of ~42 nm in diameter and are resistant to environmental inactivation (8, 31, 32; www.ncbi.nlm.nih.gov/ICTVdb/). The genome organization of the 5.1-kb circular genome is largely conserved and encodes six major proteins (20, 31, 32). The nuclear large tumor (LT) antigen and cytoplasmic small T antigen are expressed early in the viral life cycle, followed by the late cytoplasmic agnoprotein and the capsid proteins VP1, VP2, and VP3, which are transported into the nucleus for virion assembly (31, 32, 44). LT is a multifunctional regulatory protein with distinct domains (45, 49). PyV capsids are formed by the assembly of 72 VP1 pentamers into a $T=7$d icosahedral lattice associating with VP2 and VP3 and the circular viral genome (11, 35, 39).

Seroprevalence data indicate that 50 to 90% of the general population has been exposed to BKV and JC virus (25, 33, 47). Both viruses persist in the renourinary tract as the principal site of latency (13, 27). Asymptomatic reactivation is observed in 5 to 20% of healthy BKV-seropositive individuals shedding low viral loads of <5 log genome equivalents (geq)/ml urine (27). In kidney transplant patients (KTP), urinary shedding of BKV increases to 40 to 80%, with high viral loads of >7 log geq/ml. These KTP are at high risk of progression to BKV viremia and PyV-associated nephropathy (PVAN) (29, 38). PVAN has been diagnosed in up to 10% of adult KTP, with a risk of graft failure for more than 80% of cases (28, 41, 42). The emergence of PVAN has been attributed to the widespread use of more potent immunosuppressive drugs that reduce acute rejection episodes (48) but also impair BKV-specific immune control (15, 28). While the role of BKV-specific cellular immunity has been emphasized, KTP with low levels of BKV antibodies may have an increased risk of PVAN (15, 23, 46). Moreover, higher antibody titers in kidney donors have been linked to an increased risk of BKV replication in the respective recipients (10).

Despite the potential role of the BKV serostatus, the role of antibodies to different viral antigens has not been systematically studied in young KTP (1). To better understand the humoral immune response and potential correlates of protection, we compared enzyme immunoassay (EIA) responses using BKV early and late proteins purified from insect cells infected with recombinant baculoviruses. We compared anti-BKV immunoglobulin G (IgG) responses in healthy donors

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▽ Published ahead of print on 27 May 2009.

(HD) with those in KTP at a young median age and corresponding age-matched dialysis patients (DP).

MATERIALS AND METHODS

Study participants. We analyzed plasma samples from 46 KTP (young median age, 15 years; range, 3 to 32 years) and from 99 age-matched DP prior to transplantation (median age, 15 years; range, 2 to 33 years) collected between January 2002 and November 2005 in the Pediatric Nephrology Unit of the G. Gaslini Institute in Genoa, Italy. Plasma samples were also collected and analyzed from 28 HD (12 young median age donors; median age, 8 years; range, 2 to 36 years; and 16 older median age donors; median age, 39.4 years; range, 27.4 to 59 years). In KTP, the samples were collected as part of a prospective study at 1, 3, 6, 9, 12, 18, 24, and >24 months posttransplantation. At these time points, the BKV viral load in plasma and urine was determined by real-time PCR analysis (30). Maintenance immunosuppression consisted of cyclosporine A and steroids or cyclosporine A-mycophenolate mofetil and steroids in 37 KTP, tacrolimus and steroids in 3 KTP, and tacrolimus-mycophenolate mofetil-steroids in 6 KTP. The protocols were approved by the Institutional Review Board.

Construction of recombinant baculovirus transfer plasmids. Recombinant baculoviruses were generated by using the Bac-to-Bac system as described in the manufacturer's instructions (Invitrogen, Carlsbad, CA). The BKVP1 coding region (nucleotides 1564 to 2652) was amplified by PCR from urine carrying wild-type BKV by using BKVP1f1 (5′-GCGCGGATCCCCGAAAACCTGTATTTTCAGGGCATGGCCCCAACCAAAAGAAAAGA-3′) and BKVP1rev (5′-TTTTCTCGAGTTAAAGCATTTTGGTTTGCA-3′). All glutathione *S*-transferase (GST) fusion proteins contain a 21-nucleotide spacer (underlined) with a tobacco etch virus site as a potential proteolytic cleavage site. The PCR product and the pFastBac1-GST vector were digested with BamHI plus XhoI and ligated in frame to generate pFastBacGST-BKVP1. The three subdomains of LT antigen were generated by PCR using pTRELTag (24) as a template and the following primers: BKVLTD1-f1 (GCGCGGATCCCCGAAAACCTGTATTTTCAGGGCATGGATAAAGTTCTTAACAGGGAAGA) and BKV-LTD1-rev (TTTTCTCGAGTTACTTTCTTTTTTTTTTGGGTGGTGTTG); BKV-LTD2-f1 (GCGCGGATCCCCGAAAACCTGTATTTTCAGGGCATGGTAGAAGACCCTAAAGACTTTC) and BKV-LTD2-rev (AACACTCGAGTTATGGGCTAAAATCATGCTCCTTTAA); and BKV-LTD3-f1 (ATGTGGATCCCCCGAAAACCTGTATTTTCAGGGCATGGAAGAGCCTGAAGAAACAAAGC) (underlined sequences represent the tobacco etch virus site; see above) and BKV-LTD3-rev (TTTTCTCGAGTTATTTTGGGGGTGGTGTTTTAGG). The BamHI-XhoI-digested fragments BKLTD1 (amino acids [aa] 1 to 133), BKLTD2 (aa 127 to 261), and BKLTD3 (aa 262 to 695) were cloned in frame into pFastBacGST to generate pFastBacGST-BKLTD1, pFastBacGST-BKLTD2, and pFastBacGST-BKLTD3, respectively. Each PCR was carried out in a total of 50 μl containing 50 ng of plasmid DNA, 1× PCR buffer [10 mM Tris-HCl, pH 8.85, 25 mM KCl, 5 mM $(NH_4)_2SO_4$], 2.5 mM $MgCl_2$, 200 μM of each deoxynucleoside triphosphate, 1 μM of primers, and 1 U of *Pwo* DNA polymerase (Roche, Mannheim, Germany) with a temperature profile of 95°C for 4 min followed by 30 cycles of 94°C for 30 s, 55°C for 45 s, 72°C for 1.15 min, and 72°C for a 7-min extension. The identity of each plasmid DNA was verified by automated sequencing (ABI; Applied Biosystems, Foster City, CA). Recombinant baculovirus genomic DNA was prepared by transformation of vectors in *Escherichia coli* strain DH10Bac. Recombinant baculovirus stock was generated by transfection of Sf9 insect cells (at a density of 2×10^6 cells/ml) with recombinant Bacmid DNA using Cellfectin (Invitrogen). For large-scale production of proteins, 2×10^6 cells per ml of Sf9 cells were infected with recombinant baculovirus at an estimated multiplicity of infection of ~0.1 in Sf-900 II SFM medium at 28°C. To purify GST fusion proteins, infected cells were harvested at 72 h postinfection and washed once with ice-cold phosphate-buffered saline (PBS) at pH 7.4. The cells were incubated in a 1/20 culture volume of lysis buffer 100 (10 mM Tris-HCl, pH 8, 100 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 0.1% NP-40 supplemented with complete EDTA-free protease inhibitors; Roche, Basel, Switzerland) on ice for 10 min. The cells were homogenized using a glass mortar and pestle in combination with short-pulse sonification on ice followed by centrifugation at $10,000 \times g$ for 10 min, and the supernatant was collected (cytosolic lysate). The pellet (nuclei) was resuspended with a 1/10 culture volume of lysis buffer 500 (10 mM Tris-HCl [pH 8], 500 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 0.1% NP-40) supplemented with EDTA-free complete protease inhibitors (Roche) and subjected to homogenization and centrifugation as described above. The two supernatants (cytosolic and nuclear lysates) were combined and loaded onto a small column containing glutathione Sepharose 4B beads (Amersham Pharmacia, Piscataway, NJ) and incubated with gentle rocking on a shaker at 4°C overnight. The beads were washed five times with 50× gel volumes of ice-cold PBS, and the fusion proteins were eluted with 10 mM glutathione in the presence of 1 mM dithiothreitol (DTT) and complete EDTA-free protease inhibitor (Roche). The levels of protein expression in different passages and protein purity were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, Coomassie blue staining, and Western blotting using either anti-VP1 monoclonal antibody (a generous gift from C. H. Rinaldo, University of North Norway) at a 1:2,500 dilution or anti-GST monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at a 1:5,000 dilution for the detection of denatured VP1 or GST fusion proteins, respectively.

To produce viruslike particles (VLPs), the native BKVP1 coding sequence was PCR amplified using BKVP1f1 (AAGAGCGCGCCACCATGGCACCAACCAAGAGAAAGGGAGAG) and BKVP1-rev (as described above) primers and cloned into the BssHII-XhoI-digested expression vector pFastBac1 to generate pFastBacBKVP1. Recombinant baculovirus stock was generated, and infected Sf9 cells were collected and homogenized as described above. The VLPs were purified essentially as previously described (35) using the following modification. The cytosolic and nuclear lysates were combined and cleared by centrifugation at $10,000 \times g$ at 4°C for 90 min. The supernatant was centrifuged in a Beckman SW27 rotor at 25,000 rpm at 4°C for 2 h. The pellet was resuspended in 4.5 ml of buffer A (150 mM NaCl, 10 mM Tris-HCl [pH 7.4]) supplemented with EDTA-free complete protease inhibitors (Roche), incubated at 4°C overnight, and subsequently sonicated with short-pulse sonification to disrupt aggregates. After being mixed with 2.1 g of CsCl, the samples were centrifuged in a Beckman SW55 rotor at 35,000 rpm at 4°C for 24 h. Each band was harvested by puncturing the tubes with an 18-gauge needle. To remove CsCl, each band was diluted ~30× with buffer A and cenrtifuged in a Centrikon TFT 45.94 rotor at 43,000 rpm at 4°C for 4 h, and the pellet was then resuspended in buffer A supplemented with EDTA-free complete protease inhibitors (Roche) at 4°C. To determine the efficiency of VLP assembly, CsCl-purified VLPs were absorbed on Formvar-coated copper grids. The negative staining was performed with 1% phosphotungstic acid, pH 7.0, before specimens were viewed in a Philips CM100 electron microscope.

Hemagglutination inhibition assays (HIA) and EIA. HIA and EIA were performed as described previously (22, 23). For EIA, standard 96-well plates with high-level coating properties were used for coating with purified antigens at 4°C overnight (25 ng of GST fusion proteins and 50 ng of BKVLP). The wells were washed five times with 0.1% Tween 20, treated with blocking buffer (PBS, pH 7.4, 4.0% bovine serum albumin, 0.1% Tween 20) at room temperature (RT) for 2 h, and washed three times. The coated wells were incubated at RT for 1 h with 100 μl of patient sera diluted 1:400, washed five times, incubated with anti-human IgG antibody (Sigma-Aldrich, St. Louis, MO; 1:10,000 dilution in PBS, pH 7.4) at RT for 1 h, and washed again five times. The *o*-phenylenediamine hydrochloride (Sigma-Aldrich) color reaction was stopped after 30 min at RT by adding 1 N sulfuric acid. Optical densities were measured using an automated plate reader (Tecan Group Ltd., Männedorf, Switzerland) at 492 nm. Purified GST or Sf9 extract was used as a negative control and subtracted from the respective GST-BKLTD1, GST-BKLTD2, GST-BKLTD3, GST-BKVP1, or BKVLP signals, respectively. For a cutoff, an optical density at 492 nm (OD_{492}) of <0.05 was defined as nonreactive for all GST fusion proteins, and an OD_{492} of <0.11 was defined as nonreactive for BKVLP.

Statistical methods. Nonparametric statistical testes were performed using SPSS (version 14.0) to account for sample size and nonnormal distribution. OD values were treated as continuous variables, and the two-sided Mann-Whitney U test was used for calculating the *P* value. Frequency comparison of paired-antibody responses in each group was calculated by using McNemar's test. The correlation between HIA titers and EIA OD levels was evaluated by the two-sided Spearman's rho test. For multiple comparisons, Bonferroni correction and differences with *P* values of <0.05 were considered statistically significant.

RESULTS

Expression and purification of BKV proteins in SF9 insect cells. To investigate antibody responses to BKV early and late gene products, we generated recombinant viral proteins in Sf9 insect cells. We subdivided BKV large T anitigen (BKLT) into the N-terminal 133-aa domain 1 (BKLTD1), the internal 135-aa domain 2 (BKLTD2), and the C-terminal 433-aa domain 3 (BKLTD3) (Fig. 1A). The LT subdomains as well as the full-length BKVP1 capsid protein were fused in frame to GST to allow for single-step purification by glutathione-affinity chro-

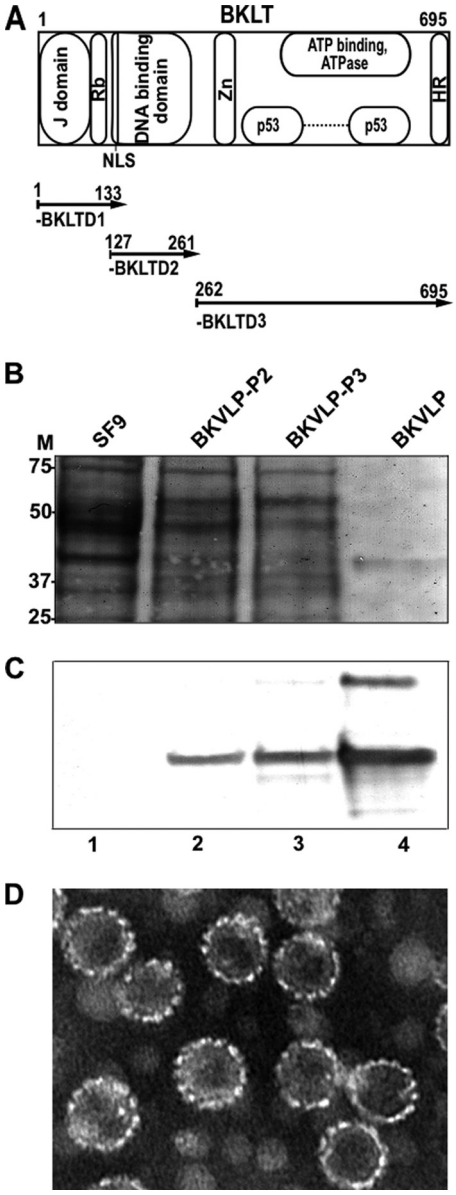


FIG. 1. Recombinant BKV proteins. (A) Schematic representation showing the linear structure of BKLT protein. (B) Purification of BKV VP1 from Sf9 cells by using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Ponceau staining of blotting membrane). Lane 1, Sf9 cells; lane 2, Sf9 cells with BKVLP in baculovirus expression vector passage 2; lane 3, Sf9 cells with BKVLP in baculovirus expression vector passage 3; lane 4, CsCl-purified BKVLP. (C) Western blotting of results shown in panel B using polyclonal rabbit anti-BKVP1 (1:2,500) antibody and goat anti-rabbit conjugated to horseradish peroxidase (1:10,000). (D) Electron microscopy and negative staining of BKVLP (magnification, ×35,000).

matography. We also expressed and purified BKVP1 without GST and generated viruslike particles (BKVLP) (Fig. 1B and C).

Correlation between HIA and EIA. Using HIA, 11 of 12 young HD (median age, 8 years) had a reactive titer of ≥20 (91.7%; median titer, 1:160). The same 11 HD were also reactive by BKVLP EIA (91.7%; median OD, 1.506) (Table 1). Using BKVP1, which as the GST fusion protein did not form viruslike particles, the HD IgG seroprevalence rate was only 83.3%, with a significantly lower median OD of 0.061 ($P < 0.05$). For the LT subdomains, the EIA responses were very low, with seropositivity rates of 25% for BKLTD1 (median OD, 0.0347), 8.3% for BKLTD2 (median OD, 0.0022), and 25% for BKLTD3 (median OD, 0.0371).

In DP (median age, 15 years; range, 2 to 33 years), all of whom were enrolled in the Pediatric Nephrology Unit of the Istituto Gaslini, BKV seroreactivity by HIA was 63.6% ($P = 0.018$) with a median titer of 1:20, significantly lower than that in HD ($P < 0.0003$; Mann-Whitney U test) (Table 1). The BKVLP rate was also significantly lower than that in HD (86.9%; median OD, 0.467; $P < 0.005$) but higher than the rate determined by HIA ($P < 0.0004$). The BKVP1 reactivity was lower than the one determined by HIA (48.5%, median OD, 0.0486). For the anti-LT responses, the following results were obtained for DP: BKLTD1 (10.1%; median OD, 0.001), BKLTD2 (9.1%; median, 0.0017), and BKLTD3 (40.4%; median, 0.0315), all with median ODs below the cutoff.

To further examine the responses in HD, we enlarged the set of tested sera to 28 HD by including 16 HD with a higher median age of 39 years (range, 27 to 59 years). The results showed an anti-BKLTD1-positive activity in 21.4% (median OD, 0.0238), an anti-BKLTD2 in 3.6% (median OD, 0.001), and an anti-BKLTD3 in 14.3% (median OD, 0.0075) of the HD. Thus, all three BKLT antigens had low OD levels as measured by EIA in both HD and DP. Comparison of EIA responses in all of the HD together revealed that the overall seroreactivity was not significantly different for BKVLP (100%), BKVP1 (93.8%), and BKLTD1 (18.8%) but that the median antibody level was lower in older median age HD for BKVLP (median OD, 0.4934; $P = 0.018$) and for BKVP1 (93.8%; median OD, 0.0173; $P < 0.001$) than in the younger HD, whereas a trend was seen only for BKLTD1 (median OD, 0.1165; $P = 0.06$).

Comparing EIA and HIA in DP, we found significant correlations between HIA and EIA levels for BKVLP ($r = 0.393$; $P < 6 \times 10^{-5}$), BKVP1 ($r = 0.344$; $P = 0.0005$), and BKLTD1 ($r = 0.270$; $P = 0.007$; Spearman's rho test), despite the presence of discordant sera which were mostly nonreactive for HIA but reactive for EIA. Although anti-BKLTD1 and anti-BKLTD2 responses were similarly low in DP (10.1% compared to 9.1%), no correlation was observed between BKLTD2 EIA or BKLTD3 EIA levels and HIA titers. We concluded that BKVLP EIA correlated well with HIA in HD and provided a more sensitive test for BKV seroreactivity in DP than HIA, BKVP1 EIA, or BKLTD1 EIA.

The difference between the anti-BKVLP and the anti-BKVP1 EIA responses likely resulted from the three-dimensional configuration of the BKVLP antigens. To address this hypothesis, BKVLP and BKVP1 were treated with 30 mM DTT, 12.5 mM EDTA, and 12.5 mM EGTA at 55°C for 60 min in order to disrupt the VLP before coating. We then examined the effect of this treatment on the seroreactivities of five patients with high levels of anti-BKVLP activity and either no concomitant activity or high levels of anti-BKVP1 activity (Fig. 2). The treatment essentially removed the BKVLP activity but had little effect on the BKVP1 response. These results indicated that the BKVLP seroreactivity was dependent on the three-dimensional conformation, as expected for antibodies

TABLE 1. BKV antibody response in HD and in DP tested by HIA and EIA[a]

Assay and protein	HD				DP				*P* value
	No. (%) of patients		Assay value[b]		No. (%) of patients		Assay value[b]		
	Neg.	Pos.	Median	IQR	Neg.	Pos.	Median	IQR	
HIA	1 (8.3)	11 (91.7)	160	80–280	36 (36.4)	63 (63.6)	20	0–80	<0.001
EIA									
BKVLP	1 (8.3)	11 (91.7)	1.5061	0.5995–1.8092	13 (13.1)	86 (86.9)	0.467	0.1517–0.9103	<0.001
BKVP1	2 (16.7)	10 (83.3)	0.061	0.0517–0.1500	51 (51.5)	48 (48.5)	0.0486	0.0062–0.0984	NS
BKLTD1	9 (75)	3 (25)	0.0347	0.0193–0.0514	89 (89.9)	10 (10.1)	0.001	0.0010–0.0133	<0.001
BKLTD2	11 (91.7)	1 (8.3)	0.0022	0.0010–0.0074	90 (90.9)	9 (9.1)	0.0017	0.0010–0.0259	NS
BKLTD3	9 (75)	3 (25)	0.0371	0.0301–0.0503	59 (59.6)	40 (40.4)	0.0315	0.0091–0.0640	NS

[a] NS, not significant; Neg., negative; Pos., positive; IQR, interquartile range. There were a total of 12 HD study participants and samples and 99 DP study participants and samples.

[b] HIA negative, titer < 1:20; HIA positive, titer ≥ 1:20. EIA antibody activity was measured as the OD_{492} at a serum dilution of 1:400. Results were as follows: BKVLP negative, $OD_{492} < 0.110$; BKVLP positive, $OD_{492} \geq 0.110$; BKVP1, BKLTD1, BKLTD2, and BKLTD3 negative, $OD_{492} < 0.05$; and BKVP1, BKLTD1, BKLTD2, and BKLTD3 positive, $OD_{492} \geq 0.05$.

directed against virions, which was not the case for the BKVP1 reactivity.

Antibody response profiles to recombinant BKV protein in KTP. To explore the BKV EIA antibody levels KTP over time, we measured the responses to all recombinant BKV antigens in three patients with different levels of BKV viruria and BKV viremia (Fig. 3).

KTP A (Fig. 3A) developed high-level BKV viruria of >7 log geq/ml and viremia of >4 log geq/ml in the first 3 months posttransplantation. Both capsid-directed antibody responses to BKVLP and BKVP1 showed a rapid rise closely following the start of BKV viruria which largely overlapped with increasing viremia. Both anti-BKVLP and anti-BKVP1 levels remained high for 24 months posttransplantation. The anti-BKLT responses were undetectable until 4 months posttransplantation. Clearing of BKV viremia and decline of BKV viruria were associated with a strong increase in anti-BKLTD1 activity, which remained high until month 24 posttransplantation. In contrast, the responses to BKLTD2 and BKLT3 remained below the cutoff level.

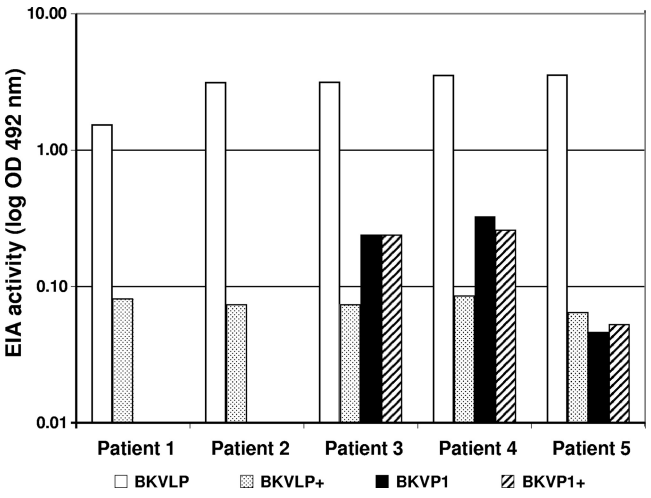


FIG. 2. Influence of BKV denaturation on EIA levels. KTP with anti-BKVLP and either without anti-BKVP1 responses (patients 1 and 2) or with anti-BKVP1 responses (patients 3, 4, and 5) were analyzed. +, treatment of indicated BKVLP or BKVP1 with 30 mM DTT, 12.5 mM EDTA, and 12.5 mM EGTA at 55°C for 1 h prior to coating to microtiter plate (see Materials and Methods).

KTP B (Fig. 3B) showed high-level BKV viruria of >7 log geq/ml and viremia of >4 log geq/ml for more than 6 months posttransplantation. Anti-BKVLP and anti-BKVP1 responses increased in parallel with BKV loads and remained high beyond the decline of BKV viremia. The anti-BKLT responses remained below the cutoff level until month 18, when anti-BKLTD1 increased to intermediate levels after clearance of viremia, whereas anti-BKLTD2 and anti-BKLTD3 reached only weakly positive levels.

KTP C (Fig. 3C) developed BKV viruria and viremia peaking at 7 log geq/ml and 3 log geq/ml, respectively, at 7 months posttransplantation. BKV viremia cleared, while BKV viruria declined to low but detectable levels. Anti-BKVLP and anti-BKVP1 increased in parallel to BKV viruria, followed by a slight but steady decline which was more pronounced for anti-BKVP1. The anti-BKLTD1 response showed weak positivity between 12 and 18 months posttransplantation, whereas anti-BKLTD2 and anti-BKLTD3 responses remained consistently below the cutoff level.

Thus, the capsid-directed antibody responses appeared to be a sensitive and rapid marker of BKV replication, with BKVLP responses being the most sensitive. In contrast, the antibody responses to the nonstructural LT appeared to increase only after more extensive BKV replication, with the BKLTD1 being the more informative antigen. We therefore restricted our further analysis to BKVLP, BKVP1, and BKLTD1 antigens.

Antibody responses in KTP. We tested 295 consecutive serum samples from 46 KTP sampled prospectively for BKV viruria and viremia. BKV viruria was detected in 33 KTP (71.7%; median, 6.14 log geq/ml) and BKV viremia was detected in 15 KTP (32.6%; median, 3 log geq/ml). BKV viruria reached a median peak level of 8.65 log geq/ml at a median of 3 months posttransplantation, whereas BKV viremia reached a median peak level of 3.9 log geq/ml at 6 months posttransplantation. All patients eventually cleared BKV viremia, which in cases of sustained positive viremia followed the reduction of immunosuppression as defined previously (22). The median antibody responses reached for an anti-BKVLP peak OD of 2.1153 at a median of 15 months posttransplantation, for anti-

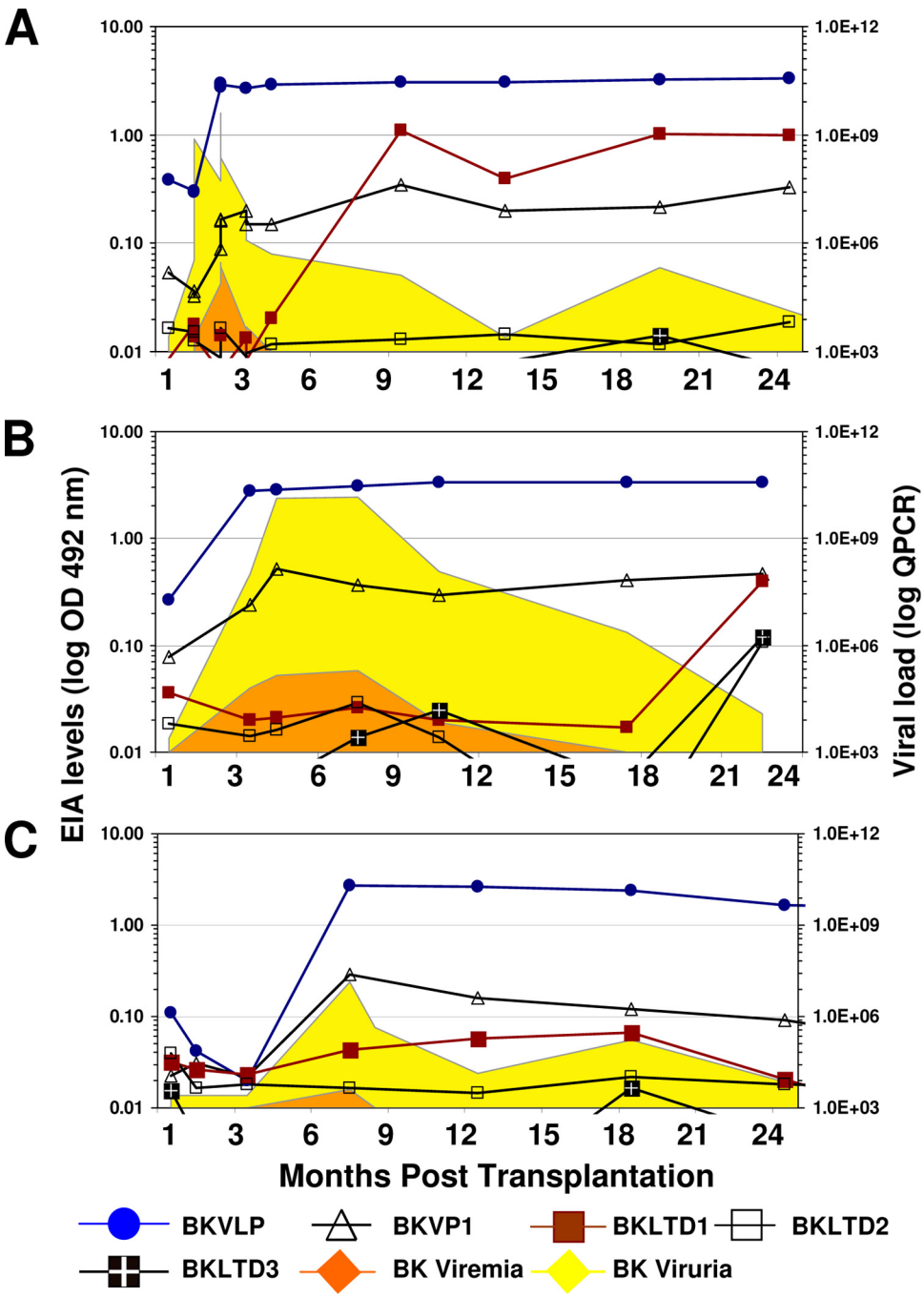
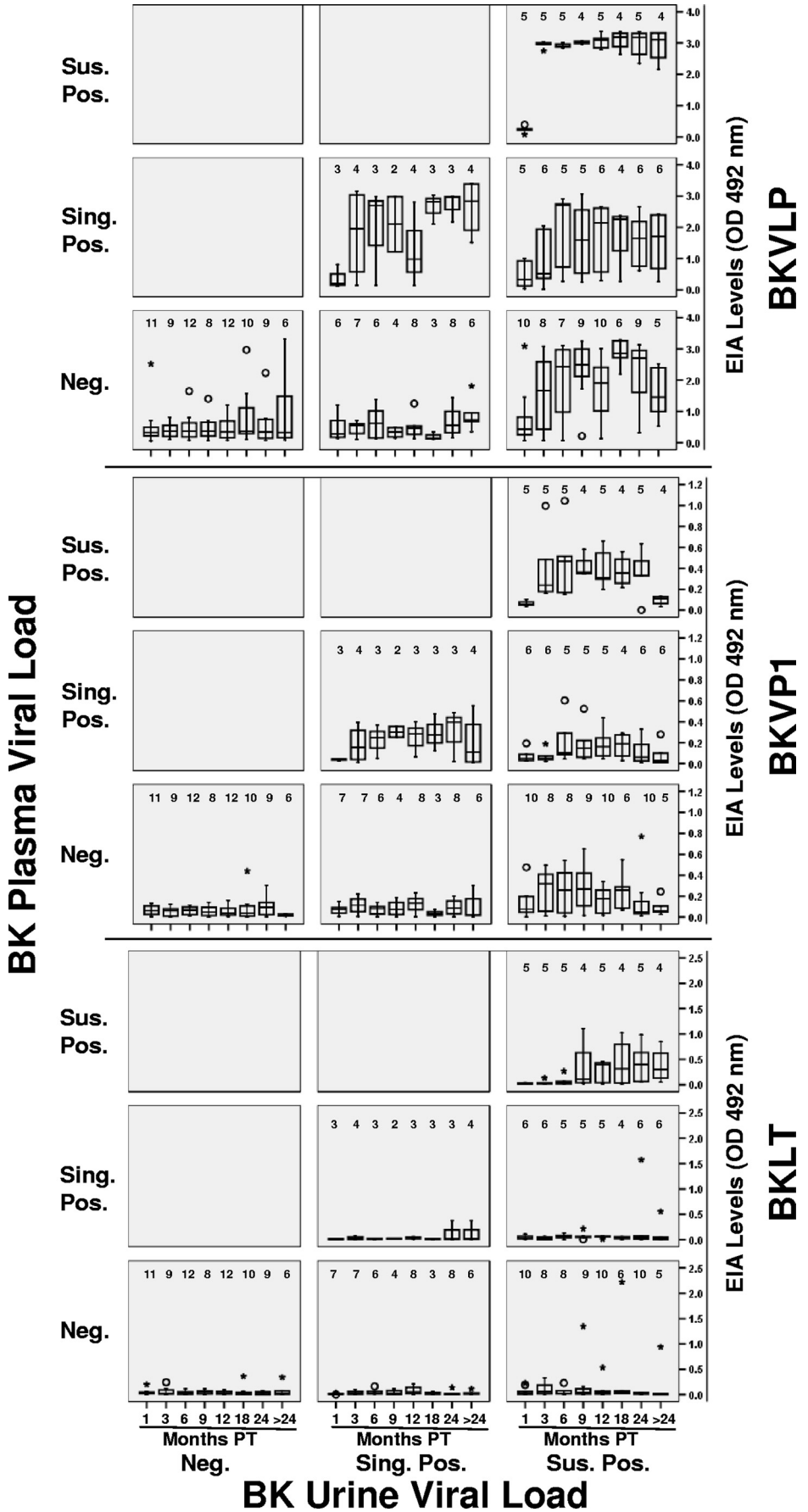


FIG. 3. BKV-specific antibody titers and viral load in selected KTP. Left *Y* axes of panels show EIA levels (log OD_{492}) for BKVLPs (blue solid circles), BKVP1 (white solid triangles), BKLTD1 (red solid squares), BKLTD2 (black solid squares), and BKLTD3 (black solid squares with white X); right *Y* axes of panels show viral load (log quantitative PCR [qPCR]) for BKV viruria (graph with yellow background) and BKV viremia (graph with orange background).

BKVP1 an OD of 0.2657 at 9 months posttransplantation, and for anti-BKLTD1 an OD of 0.1187 at 12 months posttransplantation. When the antibody kinetics in KTP were compared, anti-BKVLP antibody titers and seropositivity rates were significantly higher than that for BKVP1 or BKLTD1 ($P < 0.0001$).

To investigate the relationship between seroresponse and BKV detection in urine and plasma, we assigned the KTP into one of the following three groups: patients without detected BKV replication, patients with a single positive sample, and patients with sustained positive samples, i.e., at least two consecutively positive samples during the 3-month-interval screening (Fig. 4). We observed that theincreases in BKV-specific antibody responses were generally more pronounced in KTP with documented BKV replication and were strongest for BKVLP followed by BKVP1 and then BKLTD1 (Fig. 4). The


BK Plasma Viral Load
Sus. Pos.
Sing. Pos.
Neg.
EIA Levels (OD 492 nm)
BKVLP
BKVP1
BKLT
Months PT
1 3 6 9 12 18 24 >24
Neg.
Sing. Pos.
Sus. Pos.
BK Urine Viral Load

median antibody levels in negative, single-positive, and sustained positive groups were 0.3507, 0.8168, and 2.0695 for anti-BKVLP; 0.0565, 0.1282, and 0.2022 for anti-BKVP1; and 0.0507, 0.0307, and 0.0638 for anti-BKLTD1, respectively. The differences were also significant for anti-BKVLP and anti-BKVP1 when the BKV viruria-negative KTP group was compared with the single-positive group ($P < 0.05$) or the sustained positive group ($P < 0.001$).

The antibody responses were more pronounced in KTP with BKV viremia than in those without documented BKV viremia. The median antibody level in KTP with negative, single-positive, and sustained positive BKV viremia was 0.619, 1.8844, and 2.708 for anti-BKVLP; 0.083, 0.162, and 0.308 for anti-BKVP1; and 0.036, 0.043, and 0.127 for anti-BKLTD1, respectively. The anti-BKVLP, anti-BKVP1, and anti-BKLTD1 responses were significantly higher in KTP with sustained BKV viremia than in patients without BKV viremia, regardless of the presence or absence of BKV viruria ($P < 0.05$) (Fig. 4). Among BKV viremic KTP, those with sustained positive viremia had significantly higher anti-BKVLP and anti-BKLTD1 responses than KTP with single-positive viremia ($P < 0.01$). In fact, the anti-BK-LTD1 response was strongly correlated with the persistence of BKV viremia ($P < 0.00001$) and developed upon clearance of BKV viremia.

DISCUSSION

In this study, we report that BKV-specific IgG antibody responses differ depending on the choice of viral antigen, the degree of immunodeficiency, as well as the duration and amount of BKV exposure. The data indicate that anti-BKVLP EIA is highly sensitive to monitoring BKV-specific antibody responses, followed by anti-BKVP1 and anti-LTD1. Anti-BKVLP increased briskly even in immunosuppressed KTP who experienced only transient BKV viruria. Persistent BKV viruria as well as BKV viremia were associated with significantly higher anti-BKVLP responses remaining elevated for prolonged times posttransplantation. Although the responses to the BKV capsid antigens BKVLP and BKVP1 were significantly correlated, consistently higher IgG EIA levels were observed for BKVLP where the VP1 protein was assembled into a three-dimensional virion-like structure. The importance of the three-dimensional antigen structure was experimentally confirmed by denaturation, which caused a drop in anti-BKVLP EIA levels. Anti-BKVP1 levels were hardly affected, which indicates that different VP1-specific antibody populations may in fact coexist.

Antibody responses to BKV LT were less frequent, remained lower, and increased later than the responses to the BKV capsid protein, if at all. The relatively low anti-LT responses are remarkable, since BKV LT is an abundant viral antigen expressed early in the BKV replication cycle (4, 24, 36). Moreover, LT is conveniently detectable in the nuclei of renal tubular epithelial cells with activated BKV replication and precedes the late gene expression of agnoprotein or the capsid protein VP1 (24, 34, 44, 45). In fact, positive immunohistochemistry detecting intranuclear LT has been established as the standard confirmatory assay for the diagnosis of PVAN in renal allograft biopsies (5, 18, 28, 37, 40). The lower-antibody response despite its relative abundance suggests differences in immunogenicity. These differences may result from the protein itself, its accessibility and/or presentation to the immune system, and/or its recognition by the immune system. The last could be partly due to the fact that LT contains functional domains with high sequence homology to cellular proteins, such as the ATPase and p53 binding domains, for which a certain degree of immunological tolerance may have been established. We noted that antibody responses were not evenly distributed across the 695-aa LT protein. Antibody responses to the N-terminal domain BKLTD1 were generally higher than that to the internal or C-terminal domain. Moreover, anti-BKLTD1 showed a low but significant correlation with anti-BKVLP responses, which was not found for anti-BKLTD2 or anti-BKLTD3. Besides differences in immunogenicity, it is of interest that the N-terminal domain of LT is shared with the cytoplasmic small T antigen due to alternative splicing, which might further enhance the anti-BKLTD1 responses. Anti-BKLTD1 levels were highest in patients with a history of sustained BKV viremia, whereas sustained viruria without viremia had significantly lesser effects. Since the detection of persistent BKV viremia correlates more closely with more extensive renal allograft involvement and tubular epithelial cell loss (16, 17, 21, 24), anti-BKLTD1 may represent a serological marker of extensive BKV exposure. We also noted the correlation of rising anti-BKLTD1 titers with declining BKV loads. Thus, unlike anti-BKVLP, emerging immunological control over prolonged BKV replication is coupled to increasing LT recognition. In this sense, anti-BKLTD1 appears reminiscent of EBNA-1 antibodies directed against nuclear antigens of Epstein-Barr virus which develop 4 to 8 weeks after the antibodies to the EBV viral capsid antigen. The presence of anti-EBNA-1 has been used to identify individuals with past EBV infections, whereas the absence of anti-EBNA-1 may indicate incomplete immunological control. BKV-specific immune control has been attributed mainly to T cells which specifically recognize and lyse BKV-replicating host cells, which may be a prerequisite for developing detectable anti-BKLTD1 responses. Thus, further studies are needed to examine the hypothesis of whether or not anti-BKLTD1 responses may also serve as a specific serological surrogate marker of mounting detectable BKV-specific cellular immunity in KTP (6, 7, 14, 22).

Our study of DP indicates that BKVLP EIA are more sen-

FIG. 4. BKVx-specific antibodies and BKV viral load in plasma and urine of KTP. Antibody levels are shown as OD_{492} values on the right *y* axis for BKVLP, BKVP1, and BKLTD1; the *x* axis shows time in months posttransplantation. The KTP are grouped according the BKV DNA detection by PCR in urine (bottom) or in plasma (left) as being negative (Neg.), single-sample positive (Sing. Pos.), and sustained positive (Sus. Pos.), i.e., two or more consecutive samples positive. Box and whisker plots, with the upper and lower boundaries of the box representing 75th and 25th percentiles, respectively, and median value (line in box). Outliers are shown with the open circle, and extreme outliers are shown with a filled star above the whiskers. Numbers of samples are indicated above the blots.

sitive than HIA for determining the BKV serostatus. HIA also measures antibodies to the three-dimensional BKV capsid and has been the traditional serological method (2, 3, 33). Interestingly, no significant difference was apparent between HIA and BKVLP EIA reactivity when the seroprevalence was determined for young HD as reported previously (25). Our observation of DP is in line with the notion that chronically uremic patients have impaired immune functions. Since these patients potentially proceed to kidney transplantation, knowledge of the serostatus as a marker of BKV-specific immune memory may prove to be important to correctly identify patients at higher risk for BKV replication and nephropathy posttransplantation. We note that there are hardly any studies of young KTP that investigate viruria and viremia together with BKV-specific antibody responses with a longer follow-up of 24 months; instead, most studies are almost exclusively of adult KTP (9, 12, 26, 43).

In summary, our study emphasizes the BKVLP responses as being the most sensitive and an early serologic marker of BKV exposure compared to HIA, BKVP1, and BKVLT responses. This was true before and after transplantation and thereby extends the work of recently published studies (9, 43). Importantly, increases in anti-BKVLP responses in KTP develop during ongoing BKV viruria and viremia occurring before changes in immunosuppression and do not correlate with the curtailing of BKV replication or significant immune control as discussed previously (15). Conversely, anti-BKLTD1 responses are not a sensitive marker of BKV exposure but correlate with prolonged BKV viremia and increase with curtailing BKV replication as a potential serological surrogate of emerging BKV-specific immune control.

ACKNOWLEDGMENTS

We thank Jacqueline Samaridis and Claudia Mistl for performing the EIA and the technical team of the Molecular Diagnostic Laboratory for the BKV viral load. We also thank Georg A. Funk for advice on statistics and Isabelle Widmer for editorial assistance.

Financial support for this project was granted to H.H.H. by the Swiss National Fund grant no. 3200B0-110040/1 and by the Freie Akademische Gesellschaft Basel.

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